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14. ABSTRACT

The most significant findings here are that HerPBK10 protects siRNA from serum nuclease-mediated degradation, T7 transcribed siRNA is more cytotoxic than synthetic siRNA when delivered to HER2+ breast cancer cells by HerPBK10 in vitro, HerPBK10 directs siRNA-mediated cytotoxicity to HER2+ but not HER2- cells in vitro, the HerPBK10 carrier preferentially accumulates in HER2+ tumors in vivo when delivered systemically (intravenously), and cytotoxicity is associated with siRNA-mediated induction of IFN-alpha.

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INTRODUCTION:

The purpose of this research is to test the hypothesis that recombinant Ad5 capsid proteins targeted to HER2+ breast cancer induce tumor cell-specific death through: receptor-targeted binding and cell entry; siRNA-mediated "knock-down" of specific gene transcripts; and cytokine-mediated cytotoxicity. The main objective of this study is to demonstrate that heregulin-directed proteins target siRNA delivery to HER2+ cells in vitro and in a xenograft model of breast cancer in nude mice.

BODY:

Our first year of the project concluded with results showing that the HER2+ breast cancer –targeted carrier protein, HerPBK10, which we have previously used to target gene delivery (Medina-Kauwe et al., 2001) and corrole delivery (Agadjanian et al., 2006), stably assembles with synthetic anti-HER2 siRNA molecules and silences HER2 gene product formation in several HER2+ breast cancer cell lines.

In our second year, we demonstrated that: HerPBK10 protects siRNA from serum nuclease-mediated degradation, T7 transcribed siRNA is more cytotoxic than synthetic siRNA when delivered to HER2+ breast cancer cells by HerPBK10 in vitro, HerPBK10 directs siRNA-mediated cytotoxicity to HER2+ but not HER2- cells in vitro, the HerPBK10 carrier preferentially accumulates in HER2+ tumors in vivo when delivered systemically (intravenously), and cytotoxicity is associated with siRNA-mediated induction of IFN-alpha.

In our third year we focused on efforts to extend our findings in vivo.

COMPARING RNAi SPECIES. We first wanted to determine what type of RNAi species delivered the most potent cytotoxicity in order to incorporate it into the targeted complex and test it in vivo. We compared HER2-silencing siRNA produced either synthetically by a commercial vendor (Dharmacon), or from a T7 transcription kit (Ambion), and shRNA, which is reportedly a more effective substrate for the dicer mechanism in RNAi (communicated from collaborator). Targeted complexes or the equivalent concentration of HerPBK10 alone were added to separate sets of triplicate wells containing GFP-tagged MDA-MB-435 (HER2+) or MDA-MB-231 (HER2-) human cancer cells. At 96 hours after treatment, cell survival was assessed by convalidating assays: GFP fluorescence and metabolic activity (MTT). Both assays confirmed that the T7 transcribed siRNA is the most potent cytotoxic species (**Fig. 1**). Moreover, the HER2+ cells exhibit substantially greater sensitivity to the targeted complexes (**Fig. 1, A & C**) compared to the HER2- cells (**Fig. 1, B & D**), providing further evidence (in addition to our previous reports) that the complex is targeted to HER2+ cells.

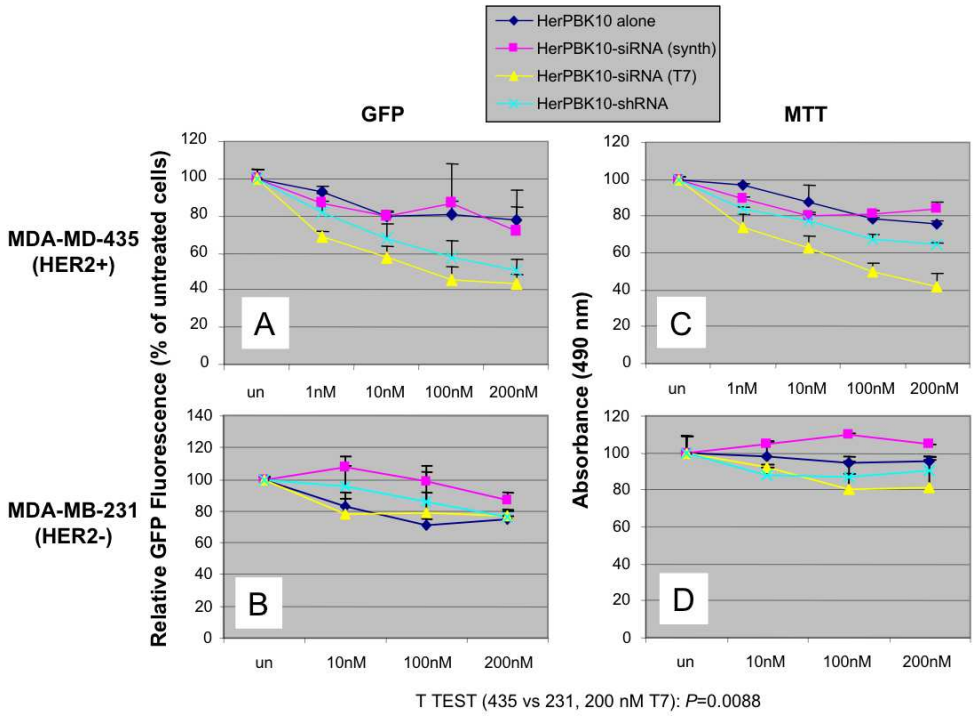


Fig. 1. Comparing RNAi Species. HerPBK10 was assembled with each RNAi species at a HerPBK10:nucleic acid molar ratio of 4 by co-incubation in 100mM Hepes+Optimem I buffer at RT for 30 min, then free RNAi species removed by ultrafiltration through a 100 mwco membrane before adding to separate wells of GFP-tagged MDA-MB-435 (HER2+; **A & C**) or MDA-MB-231 (HER2-; **B & D**) human cancer cells. Cells were assayed for GFP expression (**A & B**) and metabolic (MTT) activity (**C & D**) at 96h after treatment.

EFFECT ON TUMOR GROWTH. Our recent published studies show that HerPBK10 targets drugs to HER2+ tumor cells in vivo (Agadjanian et al., 2009). Before testing for targeted toxicity of HerPBK10-siRNA, we assessed cytotoxic potential in vivo after intratumoral (IT) injection of complexes in tumor-bearing mice. Tumor bearing nude mice received two IT injections, each a week apart from one another, of either T7 transcribed siRNA alone or HerPBK10-siRNA, and tumor volumes were measured weekly before, during, and after injections (**Fig. 2B**). Blood was also collected after each injection to assess cytokine levels (see **Fig. 3**). Mice receiving HerPBK10-siRNA exhibited an average reduction in tumor growth whereas tumor growth in mice receiving siRNA alone was unaffected (**Fig. 2, A & B**). Even though the complex was not delivered intravenously but rather IT, there still appears to be an advantage to the targeted complex. Whether this is due to enhanced tumor penetration and/or retention afforded by HerPBK10 when delivered IT, or enhanced protection from serum remains to be determined.

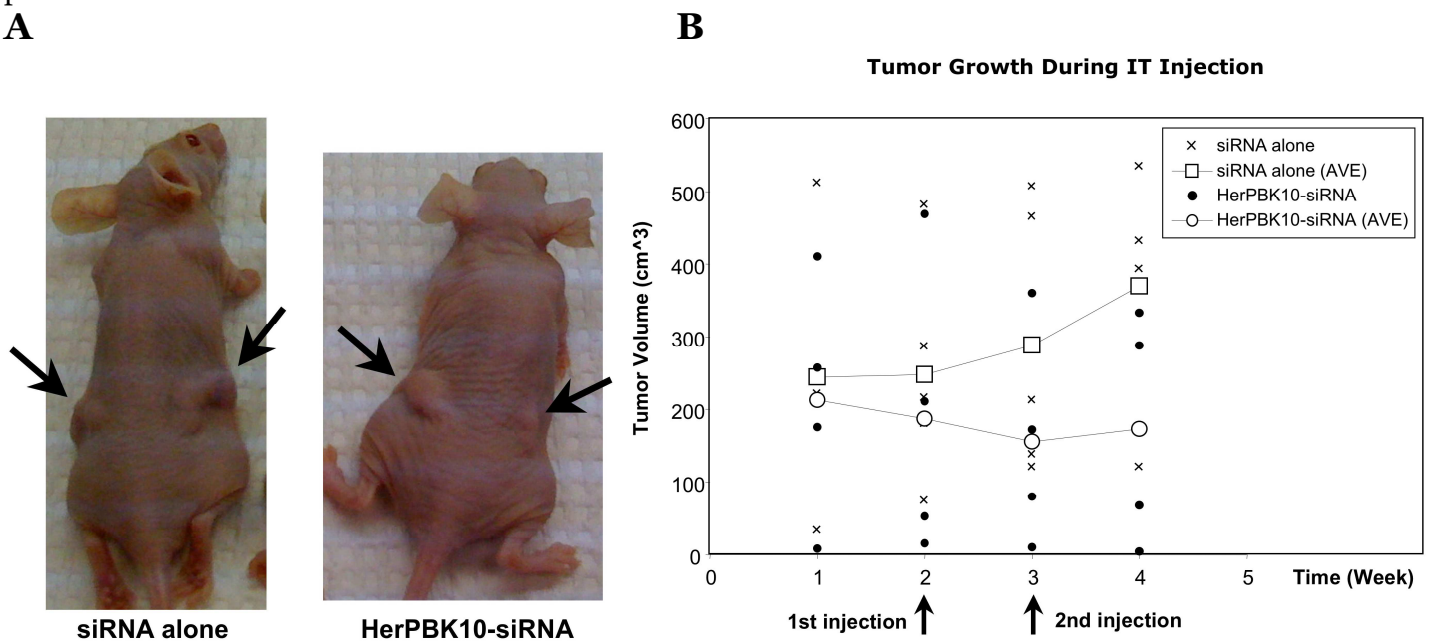


Fig. 2. Effect on Tumor Growth. Female (6-8 week) nude mice were inoculated with human HER2+ MDA-MB-435 cancer cells by subcutaneous flank injections of 1x10^7 cells per injection. At 4-6 weeks after implant, weekly measurements were taken to quantify tumor volume and track growth. Each tumor received an IT injection of either T7 transcribed anti-HER2 siRNA or HerPBK10-siRNA (1.5 nmoles final siRNA dose) on the second and third weeks of tumor monitoring. Complex was prepared as described in **Fig. 1**. **A**, representative mice at week 4 with tumors indicated by arrows. **B**, tumor volumes from mice receiving siRNA alone or HerPBK10-siRNA.

IN VIVO CYTOKINE INDUCTION. The mechanism of T7-transcribed siRNA cytotoxicity is thought to be via the induced secretion of cytotoxic cytokines as a cellular response (Kim et al., 2004). To examine whether cytokines contribute to the tumor reduction observed in **Fig. 2B**, blood was collected from the same mice at 24h after each IT injection and assayed for interferon (IFN) –alpha (our in vitro assays from the last reporting cycle show that the complexes induce IFN-alpha but not IFN-beta secretion from target cells). While overall cytokine levels remained low,

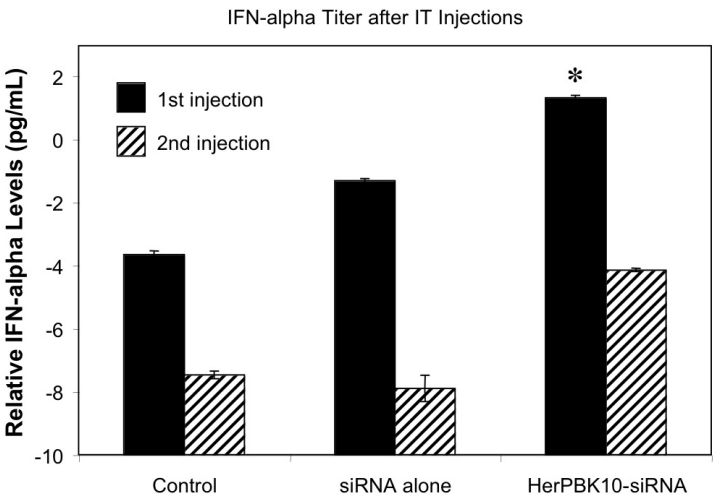


Fig. 3. In vivo cytokine induction. Blood was collected from the mice treated in **Fig. 2B** at 24h after each IT injection and relative IFN-alpha levels quantified by sandwich ELISA following manufacturer's protocol (PBL Biomedical Laboratories). Control, tumors receiving saline injections. *, P=0.05 compared to control (as determined by 2-tailed unpaired T test).

HerPBK10-siRNA injection yielded elevated circulating IFN-alpha compared to IT saline injections (control) (**Fig. 3**). The trend from the first set of IT injections indicates that siRNA alone tends to elevate circulating IFN-alpha over the controls, though not to statistically significant levels (**Fig. 3**). While our published studies show that HerPBK10 alone has no effect on HER2+ tumor cell growth (Agadjanian et al., 2009), we have yet to determine here whether the carrier protein contributes directly to cytokine elevation. It is possible that its indirect contribution results from enhanced tumor delivery, penetration, retention, but this remains to be assessed.

NEUTRALIZING ANTIBODIES. To address concerns regarding the use of an Ad capsid protein-derived carrier for siRNA, we examined whether HerPBK10 induced neutralizing antibody formation. A single inoculation of Ad can produce a long-lasting humoral response in patients (Gahery-Segard et al., 1998) and animals (Gahery-Segard et al., 1997), and prevent subsequent administration, thus reducing overall therapeutic efficacy of Ad-mediated therapies. Here we tested the antibody formation potential of HerPBK10 under the same conditions that produce an Ad capsid-elicited immune response (Yang et al., 1996).

Immunocompetent (C57BL/6) mice were inoculated with HerPBK10 at 0.05 mg/kg (with regard to protein dose) as well as a 10-fold higher dose (0.5 mg/kg). As a comparison, mice were also inoculated with Ad5 at a dose established elsewhere (Yang et al., 1996) to induce neutralizing antibodies (1.2x10^9 pfu/mouse). Blood was collected from mice before initial antigen injection, followed by blood collections every 7 days up to 35 days post- initial inoculation, while mice received a second inoculation of the same antigens on day 21 to boost any existing immunity (see **Fig. 4A, upper time chart**).

ELISA of blood serum from treated mice showed that both doses of HerPBK10 produced no significant induction of anti-HerPBK10 antibodies in comparison to untreated mice, whereas the dose of Ad5 used here triggered secretion of antibodies that recognize HerPBK10 (**Fig. 4A, graph**). As the latter result may simulate the presence of pre-existing antibodies from a previous Ad infection or exposure, it would be critical to determine whether such antibodies could prevent the binding of HerPBK10 to target cells. To test this, we assessed ligand-receptor binding by measuring the attachment capacity of MDA-MB-435 cells to HerPBK10-coated plates in immune serum. In the

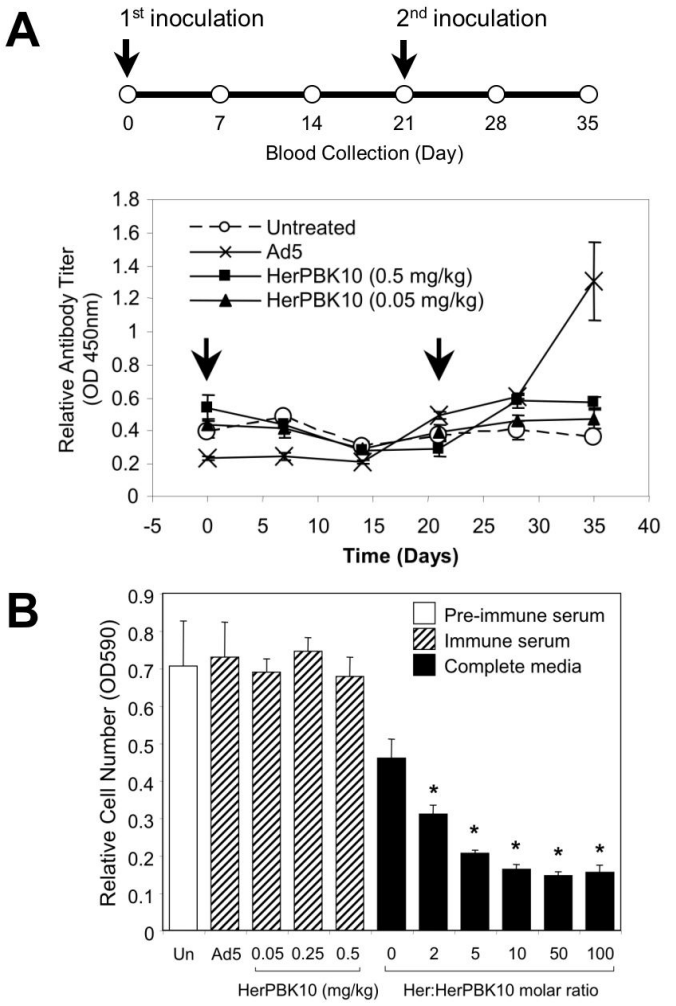


Fig. 4. Neutralizing antibody induction. **A,** Immunocompetent (C57BL/6) mice received an initial subcutaneous inoculation of HerPBK10 protein (0.05 or 0.5 mg/kg) or Ad5-GFP (1.2x10^9 pfu/mouse) followed by blood collection scheduled every seven days up to day 35 after the initial inoculation, as summarized by the **upper time chart**. On day 21, respective mice received a second inoculation of each corresponding reagent. Sera isolated from bleeds were assessed by ELISA for relative antibody titer produced against HerPBK10 (**lower graph**). Arrows denote days of antigen inoculation. N=4 mice per treatment group dose. **B**, Effect of immune sera on target cell binding. Ligand-receptor binding was tested by measuring the level of cell attachment to HerPBK10-coated plates in immune or pre-immune serum collected from mice in ***A***. Cells suspended in either pre-immune serum, immune serum from Ad5 or HerPBK10 –inoculated mice, or complete media containing 10% bovine serum but no mouse serum were incubated on HerPBK10-coated wells for 1 h at 37°C, followed by removal of free cells and measurement of attached cells by crystal violet assay. The level of receptor-specific binding was assessed on separate cells pre-incubated with competitive inhibitor (Her). Un, untreated mice. *, $P<0.01$ compared to cells attached in pre-immune serum, as determined by 2-tailed unpaired t test.

absence of serum, the cells readily attach to HerPBK10-coated wells, while the heregulin receptor-blocking ligand, Her, significantly reduces this binding, thus confirming that the cells undergo heregulin receptor-specific attachment ($P<0.05$ compared to binding without serum or competitive inhibitor, as determined by 2-tailed unpaired t test) (**Fig. 4B**). The level of cell attachment in sera from Ad5-treated mice was comparable to that in the absence of serum and in pre-immune serum (**Fig. 4B**). Likewise, sera from mice treated with 0.05 and 2.5 mg/kg HerPBK10 did not reduce attachment, and while 0.5 mg/kg HerPBK10 appeared to slightly reduce attachment, this was not significant ($P<0.05$ compared to binding without serum or competitive inhibitor, as determined by 2-tailed unpaired t test) (**Fig. 4B**).

As a side note, Ad-treated mice showed signs of inflammation and fur loss around the eye area, whereas HerPBK10-treated mice showed no such damage, and appeared similar to mock-treated mice (**Fig. 5**). Whereas such hair loss can be attributed to dominant mouse barbering or cage lesions, all Ad treated mice showed this phenotype and were continuously kept separately from other mice, whereas none of the other mice showed any such phenotype. Acute periocular hair loss and corneal damage can result from herpes simplex virus infection in mice (Smith et al., 2000) and may involve viral-induced autoimmunity (Chapman et al., 1994; Groves et al., 1995; Nagai et al., 2006). Whether Ad causes similar effects remains to be seen, but it is worth noting the correlation between Ad infection, which can induce T-cell immunity and inflammation, and the phenotype observed here.

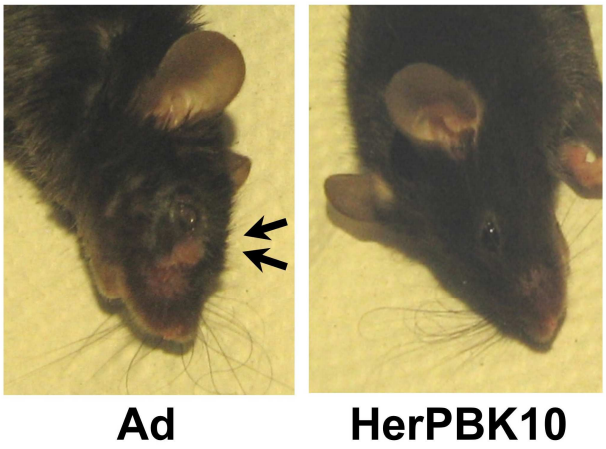


Fig. 5. External lesions in Ad vs HerPBK10 – treated mice. C57BL/6 mice inoculated with Ad or HerPBK10 as described in **Fig. 4** were observed for notable effects on eyes and periocular hair (arrows).

IN VIVO TUMOR-TARGETING OF SMALL NUCLEIC ACID PAYLOADS. We used the fluorescent DNA intercalator, doxorubicin (Dox), to tag a double-stranded oligonucleotide mimicking an siRNA molecule to track its biodistribution after systemic delivery in mice bearing 4-week old tumors (~700-800 mm^3). The Dox-intercalated nucleic acid was complexed with HerPBK10 using equivalent conditions for HerPBK10-siRNA assembly (see **Fig. 1**). Mice received a single tail vein injection of Dox alone or the complex (HerDox) (0.02 mg/kg with respect to Dox conc) and were imaged live using a customized macro-illumination and detection system. Fluorescence was evident throughout the body at 10 min after HerDox injection, then quickly accumulated at the tumors by 20 min and remained detectable in the tumors up to 100 min after injection (**Fig. 6A**). Tissues and tumors harvested at ~3h after HerDox injection showed intense

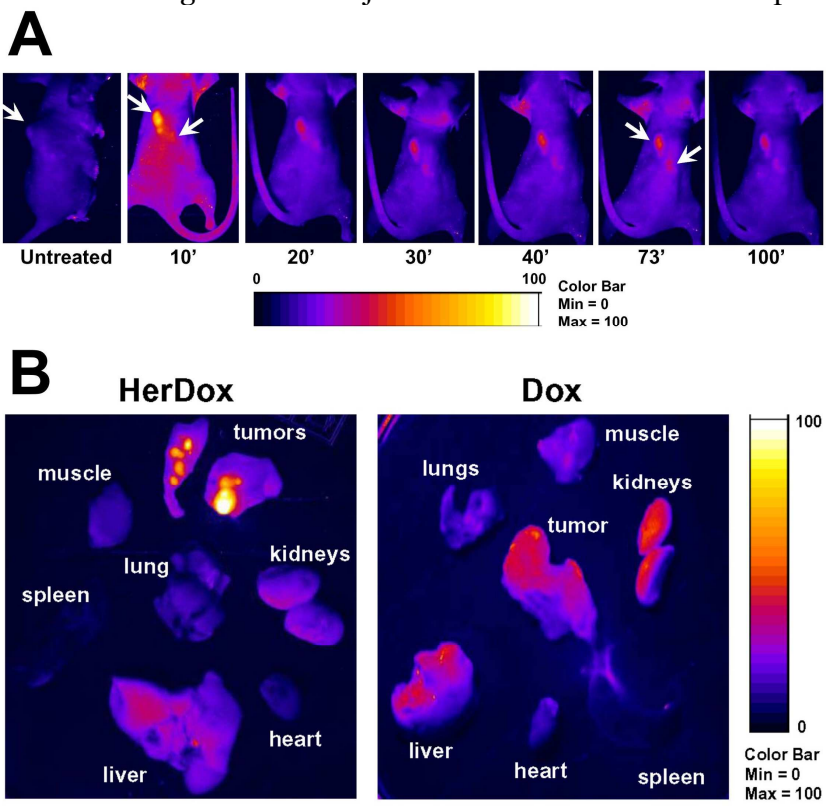


Fig. 6. Preferential targeting to HER2+ tumors. Tumor-bearing mice were injected with 0.02 mg/kg (final Dox dose) of HerDox or Dox via the tail vein and imaged with a custom small animal imager. **A**, Imaging of live mice after IV delivery of HerDox. Time points are shown as minutes after injection. Tumors are indicated by arrows. **B**, Imaging of tumors and tissues harvested at 3h after injection of HerDox or Dox. Fluorescence signal from Dox is pseudo-colored according to the color bar, with a shift toward 100 indicating high fluorescence intensity.

fluorescence in the tumors while substantially lower levels of fluorescence were detectable in the liver (**Fig. 6B**). Some fluorescence was barely detectable in the kidneys while other tissues, including the heart, spleen, lungs, and skeletal muscle, did not exhibit any fluorescence. In contrast, tissues harvested from mice injected with the equivalent dose of Dox exhibited detectable fluorescence in the liver, tumor, and kidneys. Lower levels of fluorescence were also detectable in the lungs and skeletal muscle.

TARGETING AND POTENTIAL TOXICITY RELATIVE TO HER2 LEVELS. To assess whether targeting and potency corresponds to HER2 levels, we selected lines displaying HER2 at relatively high (SKBR3), moderate (MDA-MB-435, MDA-MB-453, HeLa), and low to undetectable (MDA-MB-231) levels, according to our receptor subunit profiling of a panel of cell lines (**Fig. 7**), and performed cytotoxicity dose curves using the HerDox complex used in **Fig. 6**. We observed that HerDox CD50 inversely correlates with cell surface HER2 level on these selected lines: the cell line displaying relatively high HER2 shows a relatively higher sensitivity to HerDox whereas the cell line displaying low HER2 exhibits low sensitivity, and the lines displaying intermediate HER2 levels likewise exhibit intermediate sensitivities (**Fig. 8**; note CD50 is shown on a log scale).

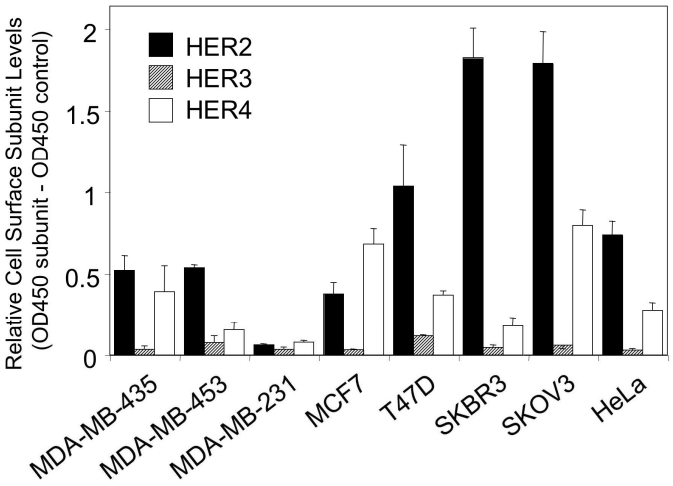


Figure 7. Relative cell surface HER subunit levels as measured by ELISA. Cells were plated on a 96-well plate at 1x10^4 cells/well and grown two days before 15 min fixation at RT with 4% paraformaldehyde in PBS, followed by rinsing 3x in PBS, and blocking with 1% BSA/PBS solution for 1h at RT. Fixed cells were incubated with anti-HER subunit antibodies (1 ug/mL anti-erbB-2/Her-2 rabbit polyclonal IgG and anti-erbB-3/Her-3 mouse monoclonal IgG from Upstate Biotechnology Inc., Lake Placid, NY, USA; and 3 ug/mL mouse monoclonal [HFR1] to Her4 from Abcam Inc., Cambridge, MA, USA), followed by incubation with HRP-conjugated secondary antibodies and enzymatic assay using standard procedures (Agadjanian et al., 2009), with product formation quantified by absorbance at 450 nm. Relative cell numbers were quantified by crystal violet staining and absorbance at 590 nm. Relative subunit levels are reported as the ELISA signal of each cell population normalized by the relative cell number, or absorbance 450 nm/ 590 nm.

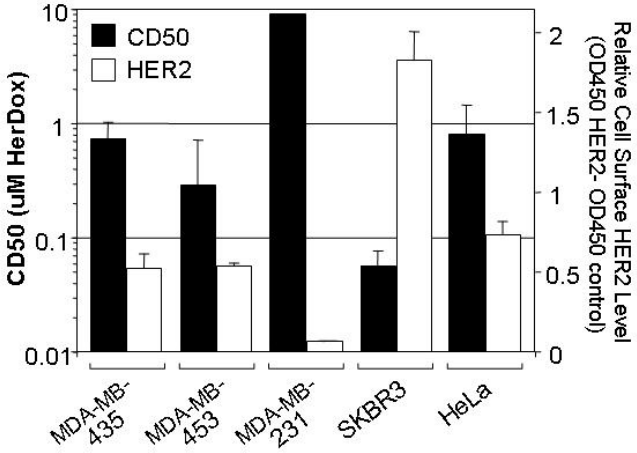
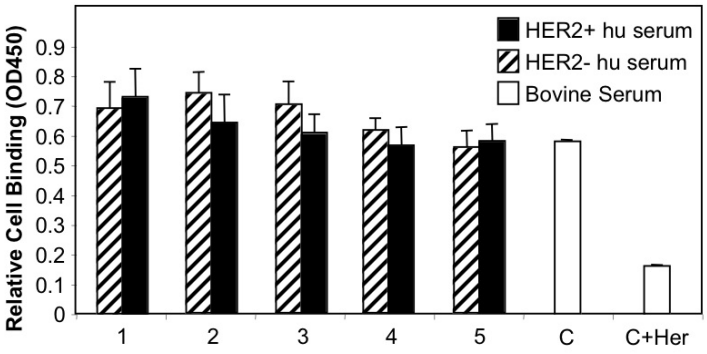


Figure 8. Toxicity to cells displaying differential HER2. Cytotoxicities from a range of HerDox doses were assessed on each cell line by metabolic assay and confirmed by crystal violet stain. CD50 values (shown in log scale) were determined by non-linear regression analyses of HerDox dose curves using a scientific graphing program (GraphPad Prism) and confirmed with an on-line calculator (http://www.changbioscience.com/stat/ec50.html). The relative HER2 level of each cell line, as obtained in Fig. S.1, is shown next to each CD50 value.

EFFECT OF PATIENT SERUM ON TARGET CELL BINDING. To determine whether HerPBK10 can compete with circulating ligand that may be present in serum, we tested HerPBK10 binding to HER2+ breast cancer cells in human serum obtained from HER2+ patients. The Women's Cancer Research Institute at Cedars-Sinai occasionally acquires limited quantities of patient serum, of which sera from HER2+ patients comprises an even smaller minority. Notably, the human serum used here is from collected whole blood of HER2+ and age-matched HER2- patients. Due to the limited quantities available for these pilot studies, we

ensured that cells received considerable exposure to the human sera (2 hours, which provides ample time for receptor binding of any circulating ligand) prior to treatment. Head-to-head comparisons of cell binding in serum from either HER2+ patients, HER2- patients, or bovine serum show no significant differences (**Fig. 9**), indicating that the human sera tested here did not interfere with HerPBK10 binding to target cells. Pre-blocking receptors with 100x competitive ligand (+Her) confirms that binding is specific to heregulin receptors.

Fig. 9. HerPBK10 binding to MDA-MB-435 cells in human serum from HER2+ or HER2- breast cancer patients. Cells were treated with HerPBK10 (1.2ug/well) in wells containing human serum from each of 5 HER2+ breast cancer patients or age matched HER2- controls, both obtained pre-chemotherapy treatment. Cells were processed for ELISA using an antibody directed at HerPBK10. Control (C) wells receiving HerPBK10 in media containing 10% bovine serum without or with 100x molar excess ligand inhibitor (+ Her) are indicated by open bars. Patient sera were provided by the WCRI tissue bank at Cedars-Sinai Medical Center. N=3 wells per treatment.



KEY RESEARCH ACCOMPLISHMENTS:

- Demonstration that T7 transcribed siRNA elicits the highest level of cytotoxicity of the different RNAi species tested for targeted tumor toxicity.
- Demonstration that HerPBK10-siRNA yields an average reduction in tumor growth compared to free siRNA, which did not reduce tumor growth.
- Demonstration that HerPBK10-siRNA elicits a small but significant elevation in IFN-alpha blood levels after IT delivery (in comparison to free siRNA, which yielded no significant increase in IFN-alpha) that corresponds to tumor shrinkage activity
- Demonstration that HerPBK10 does not elicit neutralizing antibody formation
- Demonstration that Ad5 antibodies recognize HerPBK10 but do not prevent target cell binding
- Demonstration that HerPBK10 does not elicit skin irritation effects in contrast to Ad
- Demonstration that systemically delivered HerPBK10 targets nucleic acid payload to HER2+ tumors in vivo
- Demonstration that tumor targeting and potency correlates with HER2 levels
- Demonstration that serum from either HER2+ patients or age-matched HER2- patients does not prevent HerPBK10 binding to target cells.

REPORTABLE OUTCOMES:

- Presentation of research poster at the Department of Defense Era of Hope meeting in Baltimore, MD (June, 2008)
- Invited lecture entitled “Current Research in Breast Cancer Intervention: A Message of Hope”, Third Annual Pray for a Cure Symposium and Mass with Anointing of the Sick, Cathedral of Our Lady of the Angels, October 19, 2008. Los Angeles, CA
- Invited lecture entitled “Self-Assembled Complexes for Tumor Targeted Detection and Intervention”, Biomedical and Translational Science Seminar Series, Department of Biomedical Sciences, Cedars-Sinai Medical Center, October 22, 2008. Los Angeles, CA
- Invention disclosure entitled “Targeted Delivery System for RNAi”, filed 9/24/2008
- Application to The Susan G. Komen Breast Cancer Foundation for future funding (July 2009)

CONCLUSION:

The goal of this research is to produce an improved therapeutic targeted to HER2+ tumors based on the combination of gene silencing, siRNA-induced cytokine-mediated cytotoxicity, and missile-like targeting of

these effects to tumor cell sites. The in vitro work accomplished in our previous two years demonstrate that the HER2+ targeted carrier protein, HerPBK10, targets siRNA molecules specifically to HER2+ but not HER2- cells in culture and elicits target cell death. We also demonstrated that targeted toxicity correlates with both siRNA-mediated gene silencing and induction of IFN-alpha. Here we show that HerPBK10 can target small nucleic acids, including T7 transcribed siRNA, to HER2+ tumors in vivo (in a mouse xenograft model of HER2+ breast cancer) and that tumor shrinkage correlates with IFN-alpha elevation. We also demonstrate that HerPBK10-directed targeting correlates with HER2 levels and is not inhibited by either adenovirus antiserum (which recognizes HerPBK10) or serum from HER2+ or HER2- patients. These findings suggest that it will be feasible to translate this nanoparticle into a tumor-targeted therapeutic with targeting efficacy and enhanced safety due to low off-target delivery in comparison to standard therapeutic regimens such as chemotherapy, which damages healthy tissues of the body.

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